I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL IN AN ENVELOPE ADDRESSED TO: ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231, ON THE DATE INDICATED BELOW.

BY: Lorrie Drain DATE: July 15, 2002

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent Application of Sean A. McCarthy, et al.	: Group Art Unit 1646
Conf. No.:	5645	:
Appln. No.:	09/578,063	: Examiner: Dong Jiang
Filed:	May 24, 2000	:
For:	PROTEINS HAVING DIAGNOSTIC, PREVENTIVE THERAPEUTIC, AND OTHER USES	: Attorney Docket : No. **10147-6U1** : **(MBI099-030CP1)**

#12
A.Q.J
7/31/02

REQUEST TO CORECT INVENTORSHIP PURSUANT TO 37 C.F.R. §1.48(b)

This Request is filed to correct the inventorship of the non-provisional patent application referenced above. The correct inventors were named when this application was filed. However, prosecution of the application has resulted in cancellation of claims so that fewer than all of the currently named inventors are the actual inventors of the invention being claimed in the application.

The Applicants therefore request that the application be amended by deleting the names of the following individuals as inventors for this application. Please delete:

Sean A. McCarthy

Christopher C. Fraser

There should be two co-inventors remaining (Sharp and Barnes) after entry of this Request.

07/25/2002 MAHMED1 00000040 09578063

02 FC:122 130.00 OP

The invention(s) made by the individuals who are being deleted is(are) no longer being claimed in this application.

This Request is being filed together with other papers, including an Amendment Transmittal Letter and a check in the amount of $530.00. The amount paid by check includes the processing fee set forth in 37 C.F.R. §1.17(i), believed to be $130.00.

Granting of this Request and deletion of the names of the co-inventors listed above are respectfully requested.

Respectfully submitted,

Sean A. McCarthy, et al.

15 July 2002 By: _____
(Date)

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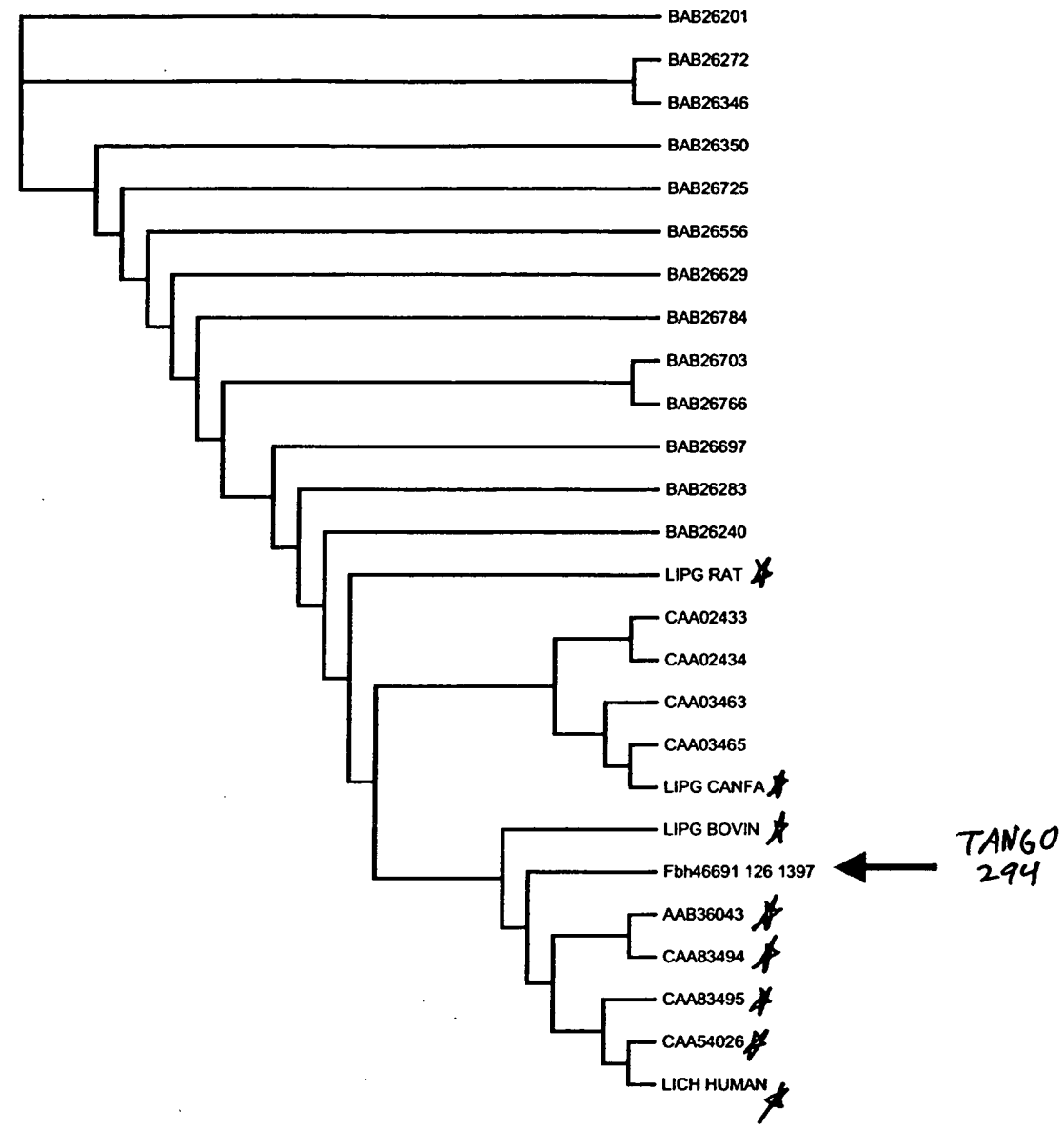
Phylogenetic tree



see Key on next page for identities of proteins.

Full BLAST Report

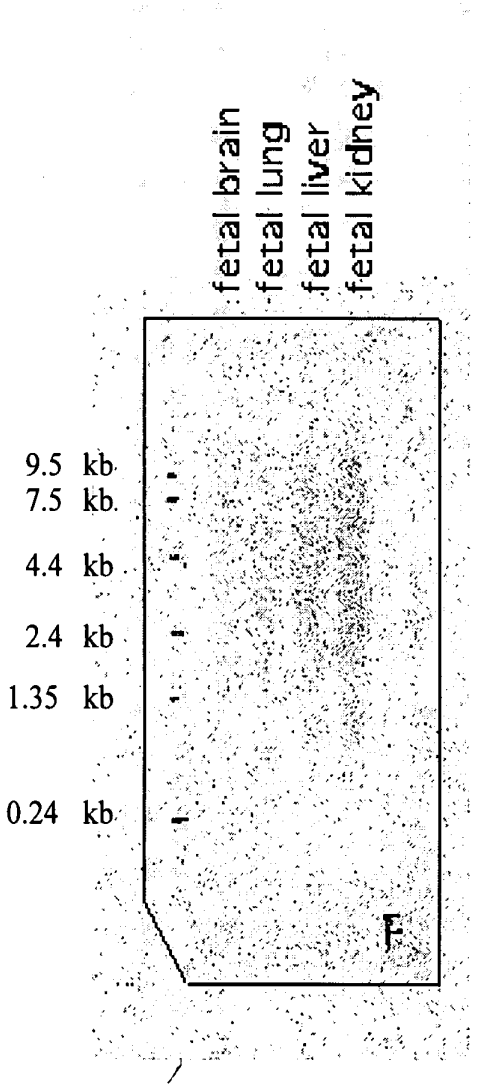
Program: BLASTP 2.0MP-WashU [07-Jun-2001] [sol2.6-ultra-ILP32F64 17:41:00 07-Jun-2001]
Query= Fbh46691_126_1397 - Import - vector trimmed
(423 letters)
Database: protot 692,327 sequences; 220,281,154 total letters.
References:

- Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215: 403-10.
- Altschul et al. (1997), Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25: 3389-3402.
- **Program Descriptions:** BLAST2 | WU-BLAST2 | Help Manual

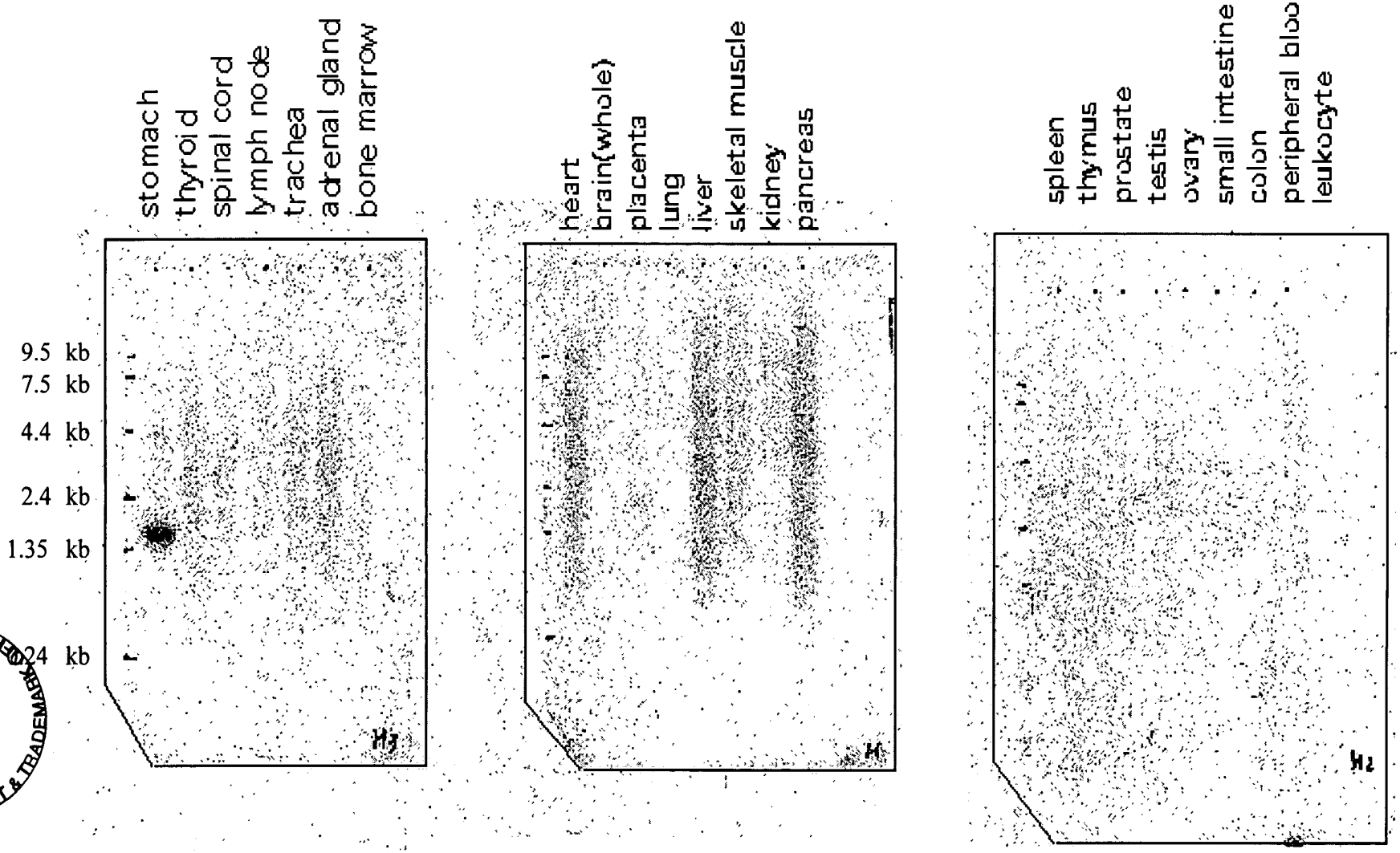
HTML formatting provided by the Bioperl Blast module.

Sequences producing High-scoring Segment Pairs:	High Score	Probability P(N)	N
emb|CAA54026| (X76488) sterol esterase [Homo sapiens] gb...	1290	2.8e-131	1
emb|CAA83495| (Z31690) lysosomal acid lipase [Homo sapien...	1290	2.8e-131	1
sp|P38571|LICH_HUMAN LYSOSOMAL ACID LIPASE/CHOLESTERYL ES...	1289	3.5e-131	1
gb|AAB36043| (S81497) lysosomal acid lipase; LAL [Rattus ...	1220	7.3e-124	1
emb|CAA83494| (Z31689) lysosomal acid lipase [Mus musculus]	1196	2.5e-121	1
sp|P04634|LIPG_RAT TRIACYLGLYCEROL LIPASE, LINGUAL PRECUR...	1166	3.8e-118	1
sp|P80035|LIPG_CANFA TRIACYLGLYCEROL LIPASE, GASTRIC PREC...	1154	7.2e-117	1
emb|CAA03463| (A57756) unnamed protein product [unidentif...	1144	8.2e-116	1
emb|CAA03465| (A57760) unnamed protein product [unidentif...	1144	8.2e-116	1
sp|Q29458|LIPG_BOVIN TRIACYLGLYCEROL LIPASE, PREGASTRIC P...	1142	1.3e-115	1
emb|CAA02433| (A39301) unnamed protein product [unidentif...	1135	7.4e-115	1
emb|CAA02434| (A39303) unnamed protein product [unidentif...	1135	7.4e-115	1
dbj|BAB26283| (AK009431) putative [Mus musculus]	1134	9.4e-115	1
dbj|BAB26556| (AK009875) putative [Mus musculus]	1134	9.4e-115	1
dbj|BAB26697| (AK010093) putative [Mus musculus]	1134	9.4e-115	1
dbj|BAB26725| (AK010139) putative [Mus musculus]	1134	9.4e-115	1
dbj|BAB26201| (AK009300) putative [Mus musculus] dbj|BAB...	1133	1.2e-114	1
dbj|BAB26272| (AK009413) putative [Mus musculus] dbj|BAB...	1133	1.2e-114	1
dbj|BAB26766| (AK010203) putative [Mus musculus]	1131	2.0e-114	1
dbj|BAB26629| (AK009990) putative [Mus musculus]	1129	3.2e-114	1
dbj|BAB26346| (AK009537) putative [Mus musculus]	1128	4.1e-114	1
dbj|BAB26240| (AK009359) putative [Mus musculus]	1127	5.2e-114	1
dbj|BAB26350| (AK009544) putative [Mus musculus]	1126	6.6e-114	1
dbj|BAB26784| (AK010231) putative [Mus musculus]	1126	6.6e-114	1
dbj|BAB26703| (AK010103) putative [Mus musculus]	1125	8.5e-114	1
dbj|BAB26359| (AK009560) putative [Mus musculus]	1124	1.1e-113	1
dbj|BAB26651| (AK010026) putative [Mus musculus]	1124	1.1e-113	1
dbj|BAB26287| (AK009437) putative [Mus musculus]	1122	1.8e-113	1
sp|P07098|LIPG_HUMAN TRIACYLGLYCEROL LIPASE, GASTRIC PREC...	1119	3.7e-113	1
dbj|BAB26733| (AK010148) putative [Mus musculus]	1119	3.7e-113	1
dbj|BAB26704| (AK010106) putative [Mus musculus]	1116	7.6e-113	1
dbj|BAB26746| (AK010173) putative [Mus musculus]	1115	9.7e-113	1
emb|CAA29414| (X05997) gastric lipase precursor [Homo sap...	1111	2.6e-112	1
emb|CAA03464| (A57758) unnamed protein product [unidentif...	1006	3.4e-101	1
emb|CAA94824| (Z70780) contains similarity to Pfam domain...	757	8.4e-75	1
gb|AAK68537|AC024835_3| (AC024835) Hypothetical protein Y5...	754	1.7e-74	1
gb|AAG45574| (AF067942) similar to lysosomal acid lipases...	753	2.2e-74	1
emb|CAB01973| (Z79696) predicted using Genefinder-Similar...	727	1.3e-71	1
dbj|BAB31766| (AK019504) putative [Mus musculus]	715	2.4e-70	1
gb|AAC69088| (AF022976) Hypothetical protein R11G11.14 [C...	706	2.1e-69	1
gb|AAC48051| (U64849) Contains similarity to Pfam domain:...	693	5.1e-68	1
emb|CAC27244| (AX068259) unnamed protein product [Homo sa...	685	3.6e-67	1
sp|O46108|LIP3_DROME LIPASE 3 PRECURSOR (EC 3.1.1.-). em...	663	7.7e-65	1
emb|CAB02896| (Z81055) Similarity to Human triacylglycero...	653	8.8e-64	1
gb|AAF52971| (AE003629) CG6113 gene product [Drosophila m...	649	2.3e-63	1
gb|AAF52985| (AE003629) CG17099 gene product [Drosophila ...	639	2.7e-62	1
gb|AAF58163| (AE003811) CG8093 gene product [Drosophila m...	629	3.1e-61	1
gb|AAC62229| (AF063014) yolk polypeptide 2 [Plodia interp...	623	1.3e-60	1
gb|AAF56528| (AE003754) CG5990 gene product [Drosophila m...	576	2.2e-55	1
dbj|BAA02091| (D12521) egg-specific protein precursor [Bo...	567	1.1e-54	1

46691 Expression in Fetal and Adult Tissues
Page 1 of 2



46691 Expression in Fetal and Adult Tissues
Page 2 of 2



or her burden or has otherwise erred in his or her position. In these situations, an examiner may have failed to set forth any basis for questioning the adequacy of the disclosure or may not have considered the whole specification, including the drawings and the written description. However, it must be emphasized that arguments of counsel alone cannot take the place of evidence in the record once an examiner has advanced a reasonable basis for questioning the disclosure. See *In re Budnick*, 537 F.2d at 538, 190 USPQ at 424; *In re Schulze*, 346 F.2d 600, 145 USPQ 716 (CCPA 1965); *In re Cole*, 326 F.2d 769, 140 USPQ 230 (CCPA 1964). For example, in a case where the record consisted substantially of arguments and opinions of applicant's attorney, the court indicated that factual affidavits could have provided important evidence on the issue of enablement. See *In re Knowlton*, 500 F.2d at 572, 183 USPQ at 37; *In re Wiseman*, 596 F.2d 1019, 201 USPQ 658 (CCPA 1979).

2107 Guidelines for Examination of Applications for Compliance with the Utility Requirement

I. INTRODUCTION

The following Guidelines establish the policies and procedures to be followed by Office personnel in the evaluation of any patent application for compliance with the utility requirements of 35 U.S.C. 101 and 112. These Guidelines have been promulgated to assist Office personnel in their review of applications for compliance with the utility requirement. The Guidelines do not alter the substantive requirements of 35 U.S.C. 101 and 112, nor are they designed to obviate the examiner's review of applications for compliance with all other statutory requirements for patentability. The Guidelines do not constitute substantive rulemaking and hence do not have the force and effect of law. Rejections will be based upon the substantive law, and it is these rejections which are appealable. Consequently, any perceived failure by Office personnel to follow these Guidelines is neither appealable nor petitionable.

II. EXAMINATION GUIDELINES FOR THE UTILITY REQUIREMENT

Office personnel are to adhere to the following procedures when reviewing patent applications for compliance with the "useful invention" ("utility") requirement of 35 U.S.C. 101 and 112, first paragraph.

(A) Read the claims and the supporting written description.

(1) Determine what the applicant has claimed, noting any specific embodiments of the invention.

(2) Ensure that the claims define statutory subject matter (i.e., a process, machine, manufacture, composition of matter, or improvement thereof).

(3) If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility if (i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (e.g., properties or applications of a product or process), and (ii) the utility is specific, substantial, and credible.

(B) Review the claims and the supporting written description to determine if the applicant has asserted for the claimed invention any specific and substantial utility that is credible:

(1) If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility.

(i) A claimed invention must have a specific and substantial utility. This requirement excludes "throw-away," "insubstantial," or "nonspecific" utilities, such as the use of a complex invention as landfill, as a way of satisfying the utility requirement of 35 U.S.C. 101.

(ii) Credibility is assessed from the perspective of one of ordinary skill in the art in view of the disclosure and any other evidence of record (e.g., test data, affidavits or declarations from experts in the art, patents or printed publications) that is probative of the applicant's assertions. An applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement.

(2) If no assertion of specific and substantial utility for the claimed invention made by the applicant is credible, and the claimed invention does not have a

readily apparent well-established utility, reject the claim(s) under 35 U.S.C. 101 on the grounds that the invention as claimed lacks utility. Also reject the claims under 35 U.S.C. 112, first paragraph, on the basis that the disclosure fails to teach how to use the invention as claimed. The 35 U.S.C. 112, first paragraph, rejection imposed in conjunction with a 35 U.S.C. 101 rejection should incorporate by reference the grounds of the corresponding 35 U.S.C. 101 rejection.

(3) If the applicant has not asserted any specific and substantial utility for the claimed invention and it does not have a readily apparent well-established utility, impose a rejection under 35 U.S.C. 101, emphasizing that the applicant has not disclosed a specific and substantial utility for the invention. Also impose a separate rejection under 35 U.S.C. 112, first paragraph, on the basis that the applicant has not disclosed how to use the invention due to the lack of a specific and substantial utility. The 35 U.S.C. 101 and 112 rejections shift the burden of coming forward with evidence to the applicant to:

(i) Explicitly identify a specific and substantial utility for the claimed invention; and

(ii) Provide evidence that one of ordinary skill in the art would have recognized that the identified specific and substantial utility was well-established at the time of filing. The examiner should review any subsequently submitted evidence of utility using the criteria outlined above. The examiner should also ensure that there is an adequate nexus between the evidence and the properties of the now claimed subject matter as disclosed in the application as filed. That is, the applicant has the burden to establish a probative relation between the submitted evidence and the originally disclosed properties of the claimed invention.

(C) Any rejection based on lack of utility should include a detailed explanation why the claimed invention has no specific and substantial credible utility. Whenever possible, the examiner should provide documentary evidence regardless of publication date (e.g., scientific or technical journals, excerpts from treatises or books, or U.S. or foreign patents) to support the factual basis for the *prima facie* showing of no specific and substantial credible utility. If documentary evidence is not available, the examiner should specifically explain the scientific basis for his or her factual conclusions.

(1) Where the asserted utility is not specific or substantial, a *prima facie* showing must establish that it is more likely than not that a person of ordinary skill in the art would not consider that any utility asserted by the applicant would be specific and substantial. The *prima facie* showing must contain the following elements:

(i) An explanation that clearly sets forth the reasoning used in concluding that the asserted utility for the claimed invention is not both specific and substantial nor well-established;

(ii) Support for factual findings relied upon in reaching this conclusion; and

(iii) An evaluation of all relevant evidence of record, including utilities taught in the closest prior art.

(2) Where the asserted specific and substantial utility is not credible, a *prima facie* showing of no specific and substantial credible utility must establish that it is more likely than not that a person skilled in the art would not consider credible any specific and substantial utility asserted by the applicant for the claimed invention. The *prima facie* showing must contain the following elements:

(i) An explanation that clearly sets forth the reasoning used in concluding that the asserted specific and substantial utility is not credible;

(ii) Support for factual findings relied upon in reaching this conclusion; and

(iii) An evaluation of all relevant evidence of record, including utilities taught in the closest prior art.

(3) Where no specific and substantial utility is disclosed or is well-established, a *prima facie* showing of no specific and substantial utility need only establish that applicant has not asserted a utility and that, on the record before the examiner, there is no known well-established utility.

(D) A rejection based on lack of utility should not be maintained if an asserted utility for the claimed invention would be considered specific, substantial, and credible by a person of ordinary skill in the art in view of all evidence of record.

Office personnel are reminded that they must treat as true a statement of fact made by an applicant in relation to an asserted utility, unless countervailing

evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. Similarly, Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.

Once a *prima facie* showing of no specific and substantial credible utility has been properly established, the applicant bears the burden of rebutting it. The applicant can do this by amending the claims, by providing reasoning or arguments, or by providing evidence in the form of a declaration under 37 CFR 1.132 or a patent or a printed publication that rebuts the basis or logic of the *prima facie* showing. If the applicant responds to the *prima facie* rejection, the Office personnel should review the original disclosure, any evidence relied upon in establishing the *prima facie* showing, any claim amendments, and any new reasoning or evidence provided by the applicant in support of an asserted specific and substantial credible utility. It is essential for Office personnel to recognize, fully consider and respond to each substantive element of any response to a rejection based on lack of utility. Only where the totality of the record continues to show that the asserted utility is not specific, substantial, and credible should a rejection based on lack of utility be maintained.

If the applicant satisfactorily rebuts a *prima facie* rejection based on lack of utility under 35 U.S.C. 101, withdraw the 35 U.S.C. 101 rejection and the corresponding rejection imposed under 35 U.S.C. 112, first paragraph.

2107.01 General Principles Governing Utility Rejections

35 U.S.C. 101. Inventions patentable

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof may obtain a patent therefor, subject to the conditions and requirements of this title.

See MPEP § 2107 for guidelines for the examination of applications for compliance with the utility requirement of 35 U.S.C. 101.

The Office must examine each application to ensure compliance with the "useful invention" or utility requirement of 35 U.S.C. 101. In discharging this obligation, however, Office personnel must keep in mind several general principles that control application of the utility requirement. As interpreted by the Federal courts, 35 U.S.C. 101 has two purposes. First, 35 U.S.C. 101 defines which categories of inventions are eligible for patent protection. An invention that is not a machine, an article of manufacture, a composition or a process cannot be patented. See *Diamond v. Chakrabarty*, 447 U.S. 303, 206 USPQ 193 (1980); *Diamond v. Diehr*, 450 U.S. 175, 209 USPQ 1 (1981). Second, 35 U.S.C. 101 serves to ensure that patents are granted on only those inventions that are "useful." This second purpose has a Constitutional footing — Article I, Section 8 of the Constitution authorizes Congress to provide exclusive rights to inventors to promote the "useful arts." See *Carl Zeiss Stiftung v. Renishaw PLC*, 945 F.2d 1173, 20 USPQ2d 1094 (Fed. Cir. 1991). Thus, to satisfy the requirements of 35 U.S.C. 101, an applicant must claim an invention that is statutory subject matter and must show that the claimed invention is "useful" for some purpose either explicitly or implicitly. Application of this latter element of 35 U.S.C. 101 is the focus of these guidelines.

Deficiencies under the "useful invention" requirement of 35 U.S.C. 101 will arise in one of two forms. The first is where it is not apparent why the invention is "useful." This can occur when an applicant fails to identify any specific and substantial utility for the invention or fails to disclose enough information about the invention to make its usefulness immediately apparent to those familiar with the technological field of the invention. *Brenner v. Manson*, 383 U.S. 519, 148 USPQ 689 (1966); *In re Ziegler*, 992 F.2d 1197, 26 USPQ2d 1600 (Fed. Cir. 1993). The second type of deficiency arises in the rare instance where an assertion of specific and substantial utility for the invention made by an applicant is not credible.

I. SPECIFIC AND SUBSTANTIAL REQUIREMENTS

To satisfy 35 U.S.C. 101, an invention must be "useful." Courts have recognized that the term "useful" used with reference to the utility requirement can be a difficult term to define. *Brenner v. Manson*, 383 U.S. 519, 529, 148 USPQ 689, 693 (1966) (simple everyday word like "useful" can be "pregnant with ambiguity when applied to the facts of life."). Where an applicant has set forth a specific and substantial

1: J Lipid Res 1996 Apr;37(4):868-76

Related Articles, Books, LinkOut

New fluorogenic triacylglycerol analogs as substrates for the determination and chiral discrimination of lipase activities.

Duque M, Graupner M, Stutz H, Wicher I, Zechner R, Paltauf F, Hermetter A.

Department of Biochemistry and Food Chemistry, SFB-Biokatalyse, Technische Universitat Graz, Austria.

A new type of fluorogenic and isomerically pure 1(3)-O-alkyl-2,3 (3,2)-diacyl glycerols was synthesized that can be used as substrate for the determination of lipase activities. These compounds contain a fluorescent pyrene acyl chain and, as a potent quencher of pyrene fluorescence, a trinitrophenylamino acyl residue. In their intact form, the fluorogens show only low fluorescence intensity. Upon lipase-induced or chemical hydrolysis of the substrates, however, the fluorophore and quencher separate from each other. This leads to a gradual increase in pyrene fluorescence, reflecting the time-dependent progress of lipolysis and, under substrate saturation conditions, lipase activity. This lipase assay is continuous and does not require separation of substrate and reaction products. Short- and long-chain homologues as well as optical isomers of the fluorogenic alkyldiacyl glycerols were hydrolyzed by pancreatic lipase, hepatic lipase, and lipo-protein lipase at highly different rates depending on the substrate or enzyme preparation and source (e.g., postheparin plasma or cultured cells). It is proposed that a useful set of enantiomeric and/or homologous substrates in combination with appropriate reaction media might be applied to the selective determination of a lipase in a mixture of lipases, e.g., hepatic and lipoprotein lipase in PHP, for medical diagnostics.

PMID: 8732786 [PubMed - indexed for MEDLINE]

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1: Anal Biochem 1994 May 15;219(1):1-8 Related Articles, Books, LinkOut

Fluorescence-based assays of lipases, phospholipases, and other lipolytic enzymes.

Hendrickson HS.

Department of Chemistry, St. Olaf College, Northfield, Minnesota 55057.

In choosing an assay, one needs to consider the following questions: What level of sensitivity is required? Must the assay be continuous? Is the substrate readily available; can it be purchased or must it be custom synthesized? How specific is the substrate? How convenient is the method? How compatible is it with monomolecular, micellar, or vesicular substrates? How tolerant is it of added detergents and proteins that may be present? What is the cost of substrates, fluorescent probes, and instrumentation? Of the many methods described in this review, discontinuous assays using natural substrates and derivatization of the products with fluorescent probes are probably the most reliable and most tolerant of reaction conditions. A drawback is the involvement of tedious and time-consuming steps which limit the number of trials that can be performed. Continuous assays, in which changes in fluorescent properties of the probe are monitored, are most convenient for kinetic studies, although they are also most sensitive to reaction conditions and intolerant of added detergents and proteins. One has to carefully consider all of these issues and choose a method best suited to the enzyme, the particular information one wants to obtain, and the availability of substrates, probes, and instrumentation. Hopefully, increased commercial availability of fluorescent substrates and probes will make these choices easier. Nevertheless, the search goes on for better, more sensitive and convenient fluorescent assays.

Publication Types:
- Review
- Review, Tutorial

PMID: 8059934 [PubMed - indexed for MEDLINE]

Display Abstract Sort Save Text Clip Add Order

1: Nahrung 1990;34(8):701-17

[The characterization of microbial lipases. 1. The determination of lipase activity]

[Article in German]

Bariszlovich M, Meusel D, Tulsner M.

Wissenschaftsbereich Lebensmittelchemie Humboldt-Universitat zu, Berlin, DDR.

In the selection of an appropriate method for activity determination of lipases existing technical equipment, kind of enzymes, number of samples investigated (e.g. in routine analysis), and expected sensitivity range have to be taken into account. Titrimetric methods and above all copper salt methods with their high detection sensitivity are the most suitable procedures for activity determination of lipases used in laboratories and institutions without equipment for radiochemical analysis.

Publication Types:
- Review
- Review, Academic

PMID: 2233988 [PubMed - indexed for MEDLINE]

2.9

SECTION IV

ANALYSIS OF DNA SEQUENCES BY BLOTTING AND HYBRIDIZATION

UNIT 2.9

Southern Blotting and Hybridization

BASIC PROTOCOL

USING NITROCELLULOSE FILTERS

Materials (see APPENDIX 1 for items with ✓)

DNA molecular weight markers (Fig. 2.5.1)
✓ 10× loading buffer
✓ 10 mg/ml ethidium bromide
0.2 N HCl
✓ Denaturation solution
✓ Neutralization solution
✓ 20×, 2×, 1×, and 0.25× SSC
✓ Prehybridization solution
Labeled probe for hybridization (*UNIT 3.5* or *3.18*)
✓ Hybridization solution
0.1% (w/v) sodium dodecyl sulfate (SDS)
Nitrocellulose membrane filters (Schleicher & Schuell #BA85)
46 × 57–cm Whatman 3MM filter paper
Sealable bags

Prepare samples and run gel

1. Digest 10 pg to 10 µg of desired DNA to completion in ~50 µl. Add 10 µl loading buffer to sample, and electrophorese on an agarose gel 12 to 24 hr (include molecular weight markers; *UNITS 3.17 & 2.5A*).

   For plasmid DNA use ≤1 µg and for mammalian total genomic DNA use 8 to 10 µg to yield signals within hours and 1 to 2 days, respectively (300-ml gel, 20 × 20 cm, with slots ~10 mm wide and 1 mm thick).

2. Stain gel 30 min in ethidium bromide (25 µl of 10 mg/ml ethidium bromide in 500 ml water), and photograph with a ruler (minimize the time the gel is exposed to UV light).

Southern transfer to nitrocellulose membrane filters

3. Treat gel as follows, covering completely and rocking gently:

   500 ml of 0.2N HCl—10 min
   Water—several times
   500 ml denaturation solution—15 min (twice)
   500 ml neutralization solution—30 min.

4. Measure gel and return to neutralization solution. Cut one piece of a nitrocellulose membrane filter such that it is 3 mm smaller in both dimensions than the gel. Wet filter in a tray of water for 1 min, then place filter into a tray of 20× SSC for 5 to 10 min.

   Always use gloves when handling nitrocellulose.

5. Cut 3 to 5 sheets of Whatman 3MM paper into pieces that are 7 mm smaller in both dimensions than the piece of nitrocellulose. Prepare a wick by cutting one piece of Whatman 3MM ~2 cm wider than the width of the gel and 30 to 40 cm long. Place several hundred milliliters of 20× SSC in a large tray, and wet wick. Put a glass plate over the tray and place wick on plate (see Fig. 2.9.1). Remove air bubbles trapped between wick and glass plate.
6. Drain gel and lay on top of Whatman 3MM wick. Remove air bubbles between gel and wick. Lay nitrocellulose exactly (do not move) on top of gel, making sure that the nitrocellulose filter does not overhang gel and remove air bubbles.
7. Wet one piece of Whatman 3MM paper in 20× SSC and place it on a piece of dry 3MM paper to remove excess SSC. Place damp piece of 3MM paper on top of nitrocellulose. Make sure that 3MM paper does not overhang nitrocellulose and remove trapped air bubbles.
8. Place stack of Whatman 3MM on top of first piece of 3MM. Put a 2- to 3-cm-thick stack of paper towels on top of the 3MM paper. Place a glass plate on top and a small bottle on glass plate.
9. Cover ends of tray with plastic wrap and allow transfer to proceed 2 to 12 hr.
10. Take apart transfer setup, and mark on filter with a blue ballpoint pen the location of slot in upper right-hand corner. Remove nitrocellulose filter and place 5 min in 2× SSC. Blot filter on a piece of 3MM paper, place between two new sheets of 3MM paper, and bake 2 hr at 80°C.

The transfer can be checked and documented by restaining and photographing the gel.

Prehybridize, hybridize, and wash filter

11. Place filter in a sealable bag, add 6 to 10 ml prehybridization solution (depending on the size of the filter), and seal bag. Prehybridize 3 hr to overnight at the desired temperature (generally 37° to 42°C.

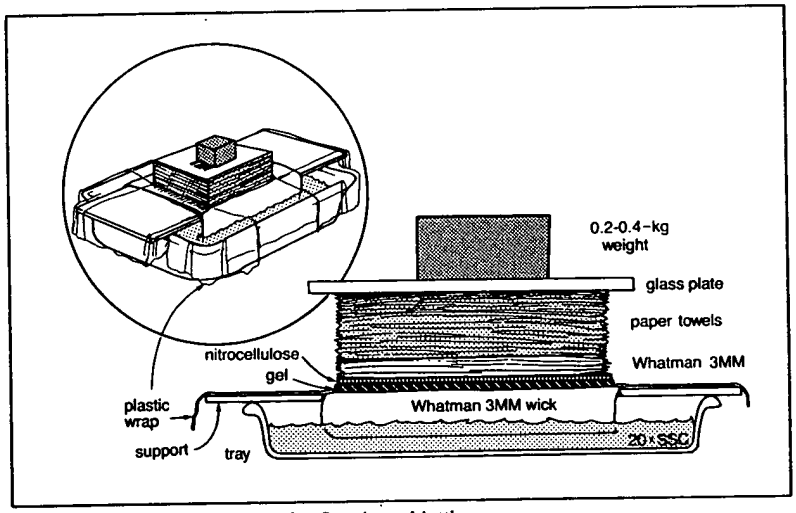

Figure 2.9.1 Transfer setup for Southern blotting.

12. Prepare probe by nick translation or other protocol (*UNIT 3.5*). Boil 500,000 cpm of probe/ml of hybridization solution to be used (6 to 10 ml) 5 min and add to hybridization solution.
13. Remove prehybridization solution, add labeled probe/hybridization solution, and seal. Hybridize 6 hr to overnight at same temperature as prehybridization.
14. Remove filter and wash with shaking as follows: 1× SSC, 0.1% SDS, room temperature (two times, each for 15 min), followed by 0.25× SSC, 0.1% SDS at hybridization temperature (two times, each for 15 min). Allow filter to air dry.

    After the low-salt washes, examine filter with a Geiger counter. If most of the filter reads at background, do not continue washing. Occasionally a more stringent wash (i.e., lower SSC concentration or higher temperature) is required.

15. Expose filter to X-ray film if radiolabeled probe was used (*APPENDIX 3*). If nonisotopic probe was used, refer to detection protocol in *UNIT 3.17*.

    For blots of moderate- or high-complexity DNA, use an intensifying screen. Bands are usually visible after the film has been exposed for 1 or 2 days. For a nick-translated probe at $\geq 10^8$ cpm/μg, 10 pg of a single-copy mammalian gene (in 10 μg of DNA) can be readily detected.

ALTERNATE PROTOCOL

USING NYLON FILTERS

Transfer using nylon filters is faster and simpler than the protocol using nitrocellulose filters. Nylon filters are also sturdier than nitrocellulose and can be reused.

Additional Materials (see APPENDIX 1 for items with ✓)

0.4 M NaOH
✓ 2×, 0.5×, and 0.1× SSC
✓ SDS/prehybridization solution
✓ SDS/hybridization solution
Nylon filters (e.g., Schleicher & Schuell, DuPont NEN, or Amersham)

1. Prepare samples and run gel as in steps 1 and 2 of basic protocol.
2. Treat gel as in step 3, omitting denaturation and neutralization treatments.
3. Cut nylon filter so that it is 3 mm smaller in both dimensions than gel and wet in water.

   Some manufacturers of nylon filters recommend that a particular side of the filter (usually the concave side) be in contact with the gel.

4. Set up the transfer pyramid as described in basic protocol (steps 4 to 9) and Figure 2.9.1, *except* use 0.4 M NaOH as the transfer solution. Transfer from 2 to 12 hr.

   NOTE: If the nylon filter is to be used for repeated hybridizations, transfer should be performed exactly as described in basic protocol (steps 3 to 10).

5. Disassemble transfer setup, mark filter (step 10), and rinse in 2× SSC for 5 min. Allow filter to air dry (do not bake).

6. Place filter in a sealable bag. Prehybridize and hybridize as in steps 11 to 13 of basic protocol.
7. Remove filter from bag and wash with shaking as follows:

   2× SSC/0.1% SDS—5 min at room temperature
   2× SSC/0.1% SDS—15 min at room temperature
   0.5× SSC/0.1% SDS—15 min at room temperature
   0.1× SSC/0.1% SDS—15 min at room temperature
   0.1× SSC/1.0% SDS—30 min at 42°C.
8. Air dry filter. If radiolabeled probe was used, expose filter to X-ray film, using an intensifying screen. If nonisotopic probe was used, refer to detection protocol in *UNIT 3.18*.

OLIGONUCLEOTIDE HYBRIDIZATION IN DRIED AGAROSE GELS

ALTERNATE PROTOCOL

In this procedure the agarose gel is dried and hybridization is carried out in situ using a radiolabeled oligonucleotide probe. Although restricted to oligonucleotide probes, the procedure is faster and more sensitive than hybridization of such probes to DNA on a membrane, and can be carried out on agarose gels containing genomic or cloned DNA.

Additional Materials (see APPENDIX 1 for items with ✓)

✓ Oligo hybridization solution
Labeled, single-stranded oligonucleotide probe (*UNIT 3.10*)
Gel dryer

1. Prepare DNA samples, run agarose gel (0.8% to 1.0% agarose, ≤5 mm thick), and photograph as described in the basic protocol.
2. Cut gel to desired size, leaving a border of at least 0.5 cm around area containing DNA samples (edges may be damaged during drying of the gel).
3. Place gel in a tray containing 4 to 5 gel volumes of denaturation solution. Rock gently 45 min.

   Do not acid-treat the gel prior to denaturation.
4. Rinse gel briefly in water, add 4 to 5 gel volumes of neutralization solution, and rock gently 30 min. Rinse gel with water, add at least 5 gel volumes of water, and rock gently 30 min.
5. Place gel on 2 to 4 layers of Whatman 3MM paper and cover with plastic wrap. Remove air bubbles between gel and plastic wrap. Place assembly on gel dryer and dry at ≤60°C until it is <1 mm thick.
6. If gel is tightly adherent to the Whatman paper, wet paper in water to release the gel. Using two dry pieces of Whatman paper as supports, slide the now fragile gel into a sealable bag, then remove Whatman paper.
7. Add oligo hybridization solution; use ≥1 ml/20 cm^2 of gel. Prehybridize 1 to 2 hr at desired temperature.
8. Prepare high-specific-activity probe by phosphorylating a single-stranded oligonucleotide using T4 polynucleotide kinase (*UNIT 3.10*).

Carry out phosphorylation reaction with ≤8 pmol oligonucleotide in a 30-µl reaction using 7000 Ci/mmol [γ-^{32}P]ATP. Separate labeled oligonucleotide from unincorporated [γ-^{32}P]ATP by centrifugation through a spin column containing Sephadex G-25 (*UNIT 3.4*).

9. Remove as much oligo hybridization solution as possible, and add minimal volume of fresh oligo hybridization solution containing 3×10^6 to 10^7 cpm probe/ml. Hybridize 3 hr at same temperature as prehybridization.

10. Remove gel and wash in a tray with 6× SSC at room temperature with gentle rocking (three times, 20 min each). Then wash at higher stringency in solution containing the appropriate concentration of SSC and 0.1% SDS (three times, 20 to 30 min each). Examine gel with a Geiger counter; no counts above background should be detectable over most of the gel.

    Generally, the final wash should be 3° to 5°C below the melting temperature of the oligonucleotide (see Table 2.9.1). Do not wash at >60°C. If higher stringency is required, lower concentration of salt in wash solution.

11. Dry gel again to same thickness (step 5), using a gel dryer or by sandwiching gel between several layers of Whatman paper and applying gentle pressure. Avoid completely drying gel as this precludes rewashing should background be excessive. Wrap in plastic wrap and expose to X-ray film.

    Detection of a single copy of the complement of the oligonucleotide in a highly complex genome will require a 2- to 3-day exposure at –70°C with an intensifying screen.

Table 2.9.1 Sample Hybridization and High-Stringency Wash Conditions for Oligonucleotide Probing of Dried Agarose Gels

Condition	20-mer oligo (10 G+C residues)	30-mer oligo (16 G+C residues)
Prehybridization/hybridization		
SSC conc.	6×	1×
Temperature	55°C	55°C
Final wash		
SSC conc.	6×	0.5×
Temperature	58°C	56°C

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Second Edition

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Published by Greene Publishing Associates and John Wiley & Sons

John Wiley & Sons

New York • Chichester • Brisbane • Toronto • Singapore

Cover art *Para I* (1959)
by Morris Louis. Collection Louisiana Museum, Humlebaek, Denmark.
Photo courtesy of the Andre Emmerich Gallery, New York.

Library of Congress Cataloging-in-Publication Data:
Short protocols in molecular biology / edited by Frederick M. Ausubel ...[et al.] —2nd ed.
p. cm.
Includes index.
ISBN 0-471-57735-9
1. Molecular biology—Technique. 2. Molecular biology—Laboratory manuals. I. Ausubel, Frederick M.
QH506.S54 1992
574.8'8'028—dc20

92-6616
CIP

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1